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REVIEW

Antibacterial peptides: basic facts and emerging concepts

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Antibacterial peptides are the effector molecules of innate immunity. Generally they contain 15–45 amino acid residues and the net charge is positive. The cecropin type of linear peptides without cysteine were found first in insects, whilst the defensin type with three disulphide bridges were found in rabbit granulocytes. Now a database stores more than 800 sequences of antibacterial peptides and proteins from the animal and plant kingdoms. Generally, each species has 15–40 peptides made from genes, which code for only one precursor. The dominating targets are bacterial membranes and the killing reaction must be faster than the growth rate of the bacteria. Some antibacterial peptides are clearly multifunctional and an attempt to predict this property from the hydrophobicity of all amino acid side chains are given. Gene structures and biosynthesis are known both in the fruit fly Drosophila and several mammals. Humans need two classes of defensins and the cathelicidin-derived linear peptide LL-37. Clinical cases show that deficiencies in these peptides give severe symptoms. Examples given are morbus Kostmann and atopic allergy. Several antibacterial peptides are being developed as drugs.

Keywords: cathelicidins, cecropins, defensins, endogenous antibiotics, innate immunity, LL-37, morbus Kostmann, peptide-based defence.

Abbreviations: Drosophila, *Drosophila melanogaster*, the fruit fly used by geneticists; Cecropia, the Cecropia moth, *Hyalophora cecropia*; NF-κB, nuclear factor κB, one of many transcription factors; IκB, inhibitor of NF-κB, destroyed after phosphorylation; κB, upstream DNA motif that binds the activated NF-κB complex; Toll, Drosophila gene, coding for a receptor, first found in development; TLR, toll-like receptors, mammalian proteins resembling toll; Imd, Drosophila gene needed for induction of antibacterial peptides; HND, human neutrophil defensins (α-defensins), sometimes only ND; HBD, human β-defesins, produced by epithelia,, sometimes only HD; Cathelicidin, group name for mammalian antibacterial peptides with the cathelin propart; LL-37, the linear C-terminal effector of cathelin-LL-37 (hCAP-18); CRAMP, the mice homologue of LL-37, a cathelicidin; PGRP, peptidoglycan recognition protein, found in most animals; LPS, bacterial lipopolysaccharide; TNF-α, tumour necrosis factor alpha; VIP, vasoactive intestinal peptide, a neurotransmitter affecting kidney function.

To be 'born with'

Most reviews in clinical journals deal with diseases and what goes wrong in the human body. This review is different: it intends to explain why most of us stay healthy. The explanation is the protection offered by the innate immune system; we share such machinery with all other animals and

also plants. The main components of innate immunity – something we all are 'born with' – are a set of peptides with antibacterial activity. These peptides – some with family names like cecropins, defensins or cathelicidins – are potent antibiotics containing some 15–45 amino acid residues and as a rule have a positive net charge. When I say 'born with' I mean it literally because the synthesis of enteric defensins in foetal tissue starts 13.5–17 weeks after gestation [1]. Moreover, a very recent report documents that LL-37 (a cathelicidin) and the defensin families are present both in Vernix Caseosa and amniotic fluid [2]. As it turns out, the two classes of defensins and LL-37 are the components most essential also for protecting our adult life from bacterial infections. As far as is known, an inherited loss of LL-37 has only been survived by four people [3]. I will return to this finding later.

What matters is the killing rate

Why did you not hear about that before? One answer is that 'innate' immunity may not be a very well chosen name – it would have been more adequate to call it 'instant' immunity, because fast killing of bacteria is a hallmark of peptide-based defence. Bacteria in the right environment can double in number every 20 min. For a surviving host it is obvious that an effective defence must be faster than the growth rate of an invader. Thus, my first illustration (Fig. 1) shows parallel *in vitro* and *in vivo* experiments in which 20 million bacteria were eliminated from the mouth of a frog. The strain of bacterium used (*Aeromonas hydrophila*) was isolated as a species normally carried by the frog and the control with a cortisone-pretreated frog shows that the frog did not swallow the bacteria [4]. It also shows that cortisone blocks *de novo* synthesis of antibacterial peptides in the frog. Our natural flora of bacteria is another reason why we need a very fast defence against bacteria. We carry nearly 2 kg microbes (mainly bacteria) in the digestive system and some 200 g on outer surfaces. The control of this flora is dependent both on the turnover of antibacterial peptides in the host and on a dynamic equilibrium in the flora itself – an ecosystem with a certain internal control. When death occurs these two mechanisms stop and the bacteria begin to multiply and brake down the dead host. It is the microbial circulation of carbon and nitrogen: 'For dust thou art and unto dust shalt thou returne' (God to Adam after the Fall, Genesis 3:19).

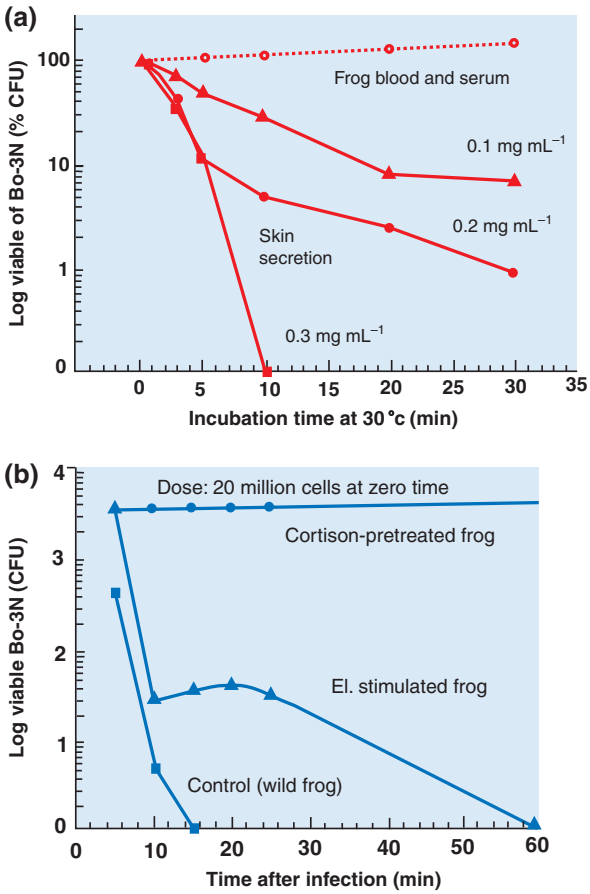

Fig. 1 Rapid elimination of frog bacteria *in vitro* and *in vivo*. A number of frog bacteria were isolated from the skin and mouth of several wild frogs, species *Rana esculenta* and *Bombina orientalis*. All animals tested carried *Aeromonas hydrophila* and strain designations were made to indicate the frog species of the isolate, in this case *B. orientalis*. (a) Different concentrations of skin secretion from the frog *R. esculenta* were used to study the rate of killing of a naladixic acid resistant mutant *A. hydrophilia* (Bo-3N) at 30 °C. The number of live bacteria at different times is given in log scale. Frog blood and serum were controls. (b) Mouth infection of three *R. esculenta* with *A. hydrophila*. One frog was untreated, one el-stimulated before the infection and one pretreated with a glucocorticoid cream on the skin. The strain used was Bo-3N and the bacterial titre in the mouth was followed by pipetting 20 μL of LB medium in the mouth and quickly withdrawing 5 μL which was plated in 3 mL soft agar on an LB plate with nalidixic acid (20 μg mL^{-1}). The results are given in log scale as per cent of the maximum value. Cortison is known to block *de novo* synthesis of all antibacterial peptides in the frog (blocking of NF-κB by induction of IκBa synthesis).

A time when nobody cared

How did 'instant' immunity start? We reported in 1972 that adult Drosophila flies could be vaccinated by an injection of harmless bacteria which protected the flies against an otherwise lethal dose of *Pseudomonas aeruginosa* [5]. The experiments indicated that the flies had a cell-free defence, but it was difficult to follow-up. We had to switch from Drosophila to pupae of a large insect. The Cecropia moth was ideal; only one generation per year and long winter hibernation (diapause) in the pupal stage. To use dormant pupae was important, because during dormancy the general metabolism of the insect is reduced to a few percent, which means that the synthesis of most proteins is shut off. Just like the flies, 'sleeping' pupae (stored in a refrigerator) could turn on their immune system after a vaccination. This gave us a significant biological enrichment of immune protein in the haemolymph (the insect blood, nearly 1 mL per pupae).

After 8 years we obtained two heat-stable peptides that killed and lysed bacteria very fast [6]. The killing reaction was insensitive to pH or salts, properties that made an enzymatic mechanism of action less likely. Sequences followed a year later and showed two linear basic peptides with 35 and 37 residues and no cysteine [7]. Cecropia has also an additional cecropin, one lysozyme and two larger antibacterial proteins, the attacins, all purified in the beginning of the 1980s.

In 1983, Lehrer and Selsted purified two antibacterial peptides with three intralinked cysteines from rabbit lung macrophages [8]. These were later named defensins [9].

When something unexpected but universal is found, fellow scientist most often say, 'Oh, it is just something special with that insect or that cell'. Thus, those involved had to continue with more peptides isolated from other insects and from granulocytes of other mammals. The interest in this field increased after Zasloff's isolation of magainins from the skin of a water frog [10]. That paper could have been the first attempt to generalize, but it was never followed-up and the idea was overshadowed by the exploration of magainin derivatives as drugs.

The first two attempts to integrate results from different animals and include antibacterial peptides in immunology were a mini-review [11] and a Ciba Symposium with recorded discussions [12]. In accord with this new perspective being accepted, the number of peptide sequences began to increase.

In January 1994 some 50 sequences were known. Today a database in Trieste contains more than 800 sequences for antimicrobial peptides and proteins from animals and plants [13]. Of these, 346 are linear peptides of which 278 can form α-helices. There are 330 peptides with S–S bridges and these include α-defensins number 54 and the β-defensins 68.

There are many specialized reviews on immunity in insects [14] or other species such as frogs [15] and mice [16]. Many reviews are related to defensins [17], epithelial host defence [18] or intestinal immunity [19, 20], and a few have broader aims [21–23]. Insect and mammalian immunity are both linked to malaria and the complex life cycle of the parasites is discussed at the end of this article.

Wake-up time

Antibacterial peptides can be grouped into five classes with different 3-D structures [21, 24]. An additional class may be added for the θ-defensins, which are circular peptides made from two genes with a novel biosynthesis [25]. However, so far the scientific interest has been focused mainly on three of the classes: (i) linear peptides free of cysteines and often with an α-helical and amphipathic solution structure, (ii) peptides with three disulphide bonds giving peptides with a flat dimeric β-sheet structure and (iii) peptides with unusual bias in certain amino acids, such as proline, arginine, tryptophan or histidine (see Fig. 2 for selected sequences of each type).

Some antibacterial peptides show both antifungal and antiviral activity and, for this reason, the peptides are often referred to as 'antimicrobial'. I have kept the term antibacterial because I believe that this function has been the driving force during evolution. Fungi usually do not cause wild mammals survival problems and in our human ecosystems they grow slowly at pH 7 and their spread is inhibited by bacteria. Of course there are peptides that have evolved to be antifungal, such as those in plants, but I have focused this review beginning with insects followed by my interest in human peptides.

There are membrane-enveloped viruses that are susceptible to antibacterial peptides, but I believe

Fig. 2 Sequences for antibacterial peptides belonging to the three main groups. The first and the third groups does not contain any cysteine residues, whilst the defensins always have three intralinked cysteine residues. Note that the bonding pattern differs between α- and β-defensins. C-terminal residues marked with an asterisk (*) are carboxy-amidated.

this to be a minor side effect of the ability of a particular peptide to destroy certain membranes. A virus that cannot replicate inside a cell will disappear, because replication requires host ribosomes and energy supply. Thus, all biologically relevant antiviral mechanisms must provide a compromise between the interest of the virus and its host. In fact, during evolution viruses may have been beneficial for species (e.g. by gene transfer), but not always for the afflicted individual.

In the Trieste database 33 entries are labelled as 'human'. The number may seem high but both cathepsin G, lactoferrin, and other precursors are given as well as several different fragments generated by proteolysis. Some of these peptides are just described, but not really studied in human. So far, well known human factors are the enzymes lysozyme and phospholipase. Then there are peptides: five α-defensins in neutrophils and Paneth cells, four β-defensins in epithelia and the cathelicidin derived LL-37 (Table 1). The two first groups contain three intramolecular disulphide bridges that are typical of defensins whilst LL-37 is a linear peptide without cysteine residues. It belongs to the cathelicidine family and is present in leucocytes, different epithelia and testis.

Chemical synthesis and mechanisms of action

Before cDNA cloning became possible, the only way to confirm the sequence of an antibacterial peptide was by chemical synthesis, followed by parallel finger printings and activity studies on different bacteria. This was first done for cecropin A [26]. As nature seldom is providing enough material, chemically synthesized peptides were used to characterize the antibacterial spectra of new peptides. It still holds that new peptides often are isolated in very small quantities and that synthesis is needed for the

Table 1 Antimicrobial peptides in man

α-defensins	
HNP1–3	Granulocytes (spleen, thymus, lung?)
HNP4	Granulocytes
HNP5	Paneth cells of the intestine, genital tract
β-defensins	
hBD-1	Skin, lung, gut (epithelial cells)
hBD-2	Skin, lung, gut (epithelial cells)
hBD-3	Skin, lung, tonsils
hBD-4	Testis, gastric antrum
Cathelicidin family	
LL-37 (proFALL, hCAP18)	Granulocytes, lung, skin, testis, gut, lymphocytes
Saposin family	
Granulysin	Cytolytic T cells, NK cells

study of their properties. Both cecropins and the α-defensins were tested against a variety of bacteria. All behaved more or less like broad-spectrum antibiotics (tetracycline was often used as a reference [21]), but as *in vivo* data accumulate such *in vitro* data have become less interesting.

A few bacteria known to produce proteolytic enzymes, like strains of Pseudomonas, Serratia and Staphylococcus, were generally found to be less susceptible or even resistant. Resistance to proteolytic enzymes could be overcome by making peptides with only D-amino acids (D-enantiomers [27]). However, the method was too expensive to be useful practically. A pair of enantiomers gave CD spectra that were mirror images of each other [27]. The interpretation is that native peptides form right-handed helices, whilst those with D-amino acids formed left-handed helices. Another approach to overcome proteolytic resistance would be to synthesize peptides with β-amino acids [28]. Whether this offers economic or biological advantages remains to be seen.

For all linear peptides investigated it is clear that bacterial membranes are the main target and lysis is clearly the end of the reaction. The only documented exception is PR-39, which stops bacterial DNA synthesis [29]. There seems to be a consensus: if a bacterium is exposed to a peptide, more and more peptide molecules dock on to the surface of the bacterial membrane. Finally, the membrane collapses when it is completely (?) covered by peptide molecules. This saturation was estimated to occur at around 1–10 billion molecules [30]. It is also clear that *in vitro* this reaction is over in a few minutes. Model membranes with and without cholesterol show that cholesterol prevents membrane damage. As this lipid is an essential component in eukaryotic membranes there is a consensus that cholesterol in most cases explains why natural concentrations of antibacterial peptides do not cause any self-damage.

The mechanisms of action of antimicrobial peptides have recently been reviewed [31, 32]. Several models for explaining the collapse have been proposed, but it is unlikely that the final answer is at hand. One difficulty is that the lytic action may not be the primary cause of death for bacteria: the point of no return could be passed before disruption of the membrane. One should remember that 60 years were required to understand that apoptosis was the ultimate mechanism of action behind the killing of bacteria by penicillin and chloramphenicol.

A novel approach to study the mechanism of action was recently published [33]. The core idea is to study the short-term alterations in transcription induced by sublethal concentrations of an antibacterial peptide. Unfortunately, 11 of 26 of the *Escherichia coli* genes tried have hitherto unknown functions. However, the technique might become a powerful tool for comparing different peptides and synergism as, e.g. between LL-37 and HBD-2.

Defensins have been made synthetically but the efforts and costs have so far limited their use as a tool to investigate their mechanism of action. Thus, today, it is fair to say that for linear peptides the bottleneck in understanding is the reaction rate and the final collapse. However, the mechanism(s) of action of defensins is hardly known at all. In most cases reported claims rest on the use of a special mutant strain of *E. coli* and synthetic peptide variations have not been tried.

Chemical synthesis has been used to make a number of designed peptides, including chimerical peptides (called hybrids) with the basic head (N-terminus) from cecropins and the hydrophobic tail (C-terminus) from an other peptide. Initially the cecropin-melittin 'hybrids' were made 26 residues or longer, but they could be shortened down to 12–15 residues without loss of activity [34]. Hybrid molecules often showed better antibiotic properties than their respective parents and one of these is included in Table 1. The sequences of peptides could be reversed without loss of activity (retropeptides with residues in reverse order) [35, 36]. This meant that neither the direction of the peptide bond, nor the turn of the helix (the D-enantiomers) did interfere with the membrane-lytic mechanism of the peptides. The goal was to understand more about mechanism of action, but occasionally there was a hope for an efficient drug. Mechanisms of action and synthetic peptides have been well covered in reviews [22, 31] and my consensus given here is close to that of these reviewers.

Is there a common denominator for antibacterial peptides? In the past most authors have agreed on a positive net charge (to facilitate binding to bacterial phospholipids) and on an element of amphipathicity with a hinge that will help the molecule to 'flip' into a bacterial membrane. These criteria are rather general and they fit groups of other polypeptides like histones and angiotenins, which also often have antibacterial activity. Therefore it is reasonable to ask: is there a way to distinguish the antibacterial (antimembrane) activity from the potential for hormone action?

An attempt to do this is presented in Table 2 in which the last column gives calculated values for potential protein interaction (index). The sequences for the 10 peptides are included in Fig. 2. All have antibacterial activity and some of them are also multifunctional with hormone like activities. Vaso-active intestinal peptide (VIP) is a neurotransmitter that happens to be antibacterial. All the peptides have a positive net charge, but with threefold variations. However, the per cent of positively charged residues gives a more coherent picture, but it is incomplete because there are no accounts of residues with carboxyl group and those with potential for the weak interactions. To calculate an index for binding potential one could use published thermodynamic solubility properties for amino acid side chains [37]. The sum of the solubility values for all residues in a sequence might give an overall estimate of the potential of a peptide to bind to other proteins such as different receptors. To normalize such a sum it would have to be divided by the number of residues: this was carried out for the index in Table 2. End group charges normally cancel, but not with a C-terminal amidation. Strictly speaking, neither proline residues nor amidations are side chains and they are disregarded in Table 2. For PR-39 with 18 proline residues this may be somewhat misleading.

There are three peptides namely LL-37, PR-39 and VIP with index values 2.48 or higher. They are predicted to have a high binding potential. VIP is regarded as a neurotransmitter and the multifunctional data for the other two would justify also regarding these peptides as potential hormones.

Table 2 Index of binding potential for peptides with antibacterial activity

Peptide	Residues	Net charge	% Basic	Index
LL-37	37	–8	29.7	3.00
PR-39	39	–10	25.6	3.04
CRAMP	38	–7	26.3	1.59
Cecropin P1	31	–5	22.6	1.80
Magainin 2	23	–4	17.4	0.42
Ca(1–7)M(2–9)	15	–5	33.3	–0.54
HNP-1	30	–3	13.3	1.07
HBD-2	41	–7	17.1	0.90
HBD-3	44	–11	29.5	0.90
VIP	41	–4	17.9	2.48

Index (for protein-binding potential) is the sum of the free energies of the respective side chains for transfer from cyclohexane to water taken from Radzeka and Wolfenden [37] and divided by the total number of residues. The calculated values are negative (except for the hybrid peptide), but the + and − are reversed. The per cent of positively charged residues is designated % basic. To calculate the index, we have developed a simple program named 'Molpep 3.5' which is freely available online at http://www.ki.se/jim. The sequences for all peptides are given in Fig. 2.

The cecropin-melittin hybrid CA(1–7)M(2–9) was designed only to be antibacterial and it has a negative index (−0.54). Of the natural peptides magainin has the lowest index (0.42). It predicts a low potential for interaction with receptors and the frog may not need any signalling from its effector. Thus, a low index value may indicate an antibacterial drug candidate without many side-effects. Bacterial membranes do contain 'islands' of proteins, but phospholipids with or without carbohydrate chains are the bulk material. As the binding of peptides to bacterial membranes involves the lipids rather than proteins, the index should be unrelated to the bactericidal effects of the peptides.

The index values for human defensins, HNP-1, HBD-2 and HBD-3 are rather similar (1.07, 0.90 and 0.90, respectively). A significant part of the binding potential for all defensins is probably tied up by their internal triple-stranded β-sheet structure and their ability to form dimers, all in agreement with published solution structures for human β-defensins [38].

The index values for cecropin P1 (1.80) and CRAMP (1.59) are in the middle range. We now know that cecropin P1 is produced by the nematode Ascaris living in the small intestine of pig and human. It may well be able to fulfil its function without much signalling. A knockout mouse in the CRAMP gene shows that the peptide protects the mice skin against infections, but no other function is yet described [39].

The two peptides, PR-39 and LL-37, have higher index values (3.04 and 3.00, respectively) than the neurotransmitter VIP (2.53) and there are many reports that these peptides are multifunctional. LL-37 and CRAMP are both derived from cathelin precursors and they do have certain similarities in sequence (Fig. 2), but the difference in their index values is rather large (3.00 vs. 1.59). LL-37 is unique to humans and CRAMP is unique to mice: in both cases these are derived from the single cathelicidin gene in the genome. Cell culture experiments may give further clues about the functional differences between these two peptides.

In summary, there is a relatively good correlation between the index in Table 2 and the known properties of the respective peptides. However, for HBD-3 the index may be misleadingly low. The difference in index between LL-37 and CRAMP was unexpected as they are two rather similar molecules – but perhaps the differences between men and mice is greater than often assumed by scientists.

cDNA cloning gave precursors

It was unexpected that it should be more difficult to clone smaller proteins than larger ones – but in a haystack it may be easier to find a screwdriver than a small screw. After 2 years, a cecropin B clone was found in 1985 and the cDNA sequence indicated a minor error in the C-terminus for the peptide structure. The peptide with an altered C-terminus was synthesized and by finger printing and mass analysis shown to be the same as the native product [40].

Lehrer's group had isolated defensins from a number of species and they selected human defensins for their first cloning that was reported in 1988 [41]. For all antibacterial peptides the cloning work was important because this both confirmed sequences and provided conceptual news. All peptides are made from precursor genes that also are coding for a signal peptide. Moreover, for some peptides, post-transcriptional modifications take place at both ends. An N-terminal Glu-residue might become cyclicized to pyroglutamate (that blocks the Edman reaction) whereas amidation at C-terminus is obtained by an amino group from a penultimate glycine residue, followed by a basic residue. With this information at hand, two cecropin precursors were synthesized and then processed *in vitro* [42]. It was found that the signal peptidase requires the full length of the signal, whilst the dipeptidyl aminopeptidase is a proline-specific enzyme. The antibacterial activities of analogues with truncated N-termini were investigated with and without processing enzymes. It was found that the propart of the precursors could block the antibacterial activity.

The ordered cleaving of precursors is a very important activation step, and is different for different peptides or peptide families. From the procecropins two dipeptides were removed stepwise by a dipeptidyl peptidase purified from Cecropia haemolymph. The cleavage sites in the proregion were determined with a synthetic procecropin having a proregion with two labelled proline residues with an unlabelled residue in between. The manual Edman sequencing of this peptide gave radioactivity in steps two and four. Using high-performance liquid chromatography (HPLC) analysis during the processing we could resolve the precursor, the intermediate

(with one dipeptide removed) and the end product, the mature cecropin [42]. With a larger propeptide the same mechanism is used for the activation of the bee venom toxin melittin (see review [43]).

The first few genes and their harvest

At that time genomic sequences were made manually and in Cecropia the genes were large because the introns were more than 10 times larger than the two exons. The first gene structure solved for an antibacterial peptide was the one for cecropin B in Cecropia [44]. As it was the first gene of this type, there was nothing to compare it with. We could see that there were two exons and a large intron. Upstream there was a Cat box, a TATA box and a Cap site and downstream a PolyA site but that was all.

The cloning of two more cecropin genes and the arrangement of a 20-kB cecropin cluster gave two surprises. The introns in the genes for cecropin A and cecropin D both contained movable retro elements, with inverted repeats (Mariner) or direct repeats [45]. Horizontal gene transfer is becoming accepted as part of our evolution [46], but whether or not cecropin genes have moved horizontally is an open question. There may never have been selection pressure for human cecropins because 100 years ago most humans harboured two cecropin producers.

One was the stomach bacterium *Helicobacter pylori* where the N-terminal sequence of ribosomal protein L1 was found to be cecropin-like. Two synthetic peptides with N-terminal residues were found to be potent antibacterial agents that *H. pylori* itself was resistant to [47, 48]. Antiserum raised against the synthetic peptides with residues 2–20 could identify natural peptides in HPLC fractions of *H. pylori* extracts. *In vivo*, such N-terminal peptides could counter-balance Lactobacteria competing with *H. pylori*.

The second case was the presence of nematodes. Three potent antibacterial peptides were isolated from two parasitic nematodes *Ascaris lumbricoides* and *Ascaris suum* which as five instar larvae (about 10 cm long) live in the digestive system of humans and pigs. The pig was one of the first animals domesticated by *Homo sapiens* [49] and it is likely that some 10 000 years ago our ancestors and their pigs shared both natural flora and antibacterial peptides controlling the flora.

From defensins to epithelial protection

Three findings around 1990 changed the perspective on defensins: the cloning of the α-defensins, the finding of insect defensins and the discovery of the β-defensins. The two defensin families are very similar in 3-D structures and in functions, in fact they are almost interchangeable in nature. The neutrophils of most mammals have granules containing α-defensins, but bovine neutrophiles have granule packed with β-defensins [50]. The α-defensins are made constitutively and do not seem to be induced whilst most β-defensins investigated are inducible. The α-defensins have evolved to work inside a phagosome, they have propeptides, but in the granule they are processed. If they are released their cytotoxic properties can hurt the host. The β-defensins, like TAP in bovine airways [51], have signal peptides but no propeptides (made for excretion) and they seem to be produced by all epithelial cells. They are important for the protection of mucosa both in the lungs and in the digestive system [52].

Structurally, defensins are flat triple-stranded molecules held together by three intramolecular disulphide bonds (Fig. 2). The differences between the two groups are the ways the cysteine residues are linked and the fact that the β-defensins generally are slightly larger and some have modified termini. The 3-D structure of both types of defensins were determined by X-ray diffraction and NMR. From the first X-ray work it is clear that defensins are packed together as dimers [53]. This structure almost foreboded the important finding of the θ-defensins [25]; these are small circular molecules (all peptide bonded amino acids) made from two separate genes, producing two separate defensins, finally linked covalently. However, whilst the α- and β-defensins are triple-stranded β-sheets, the θ form is only double-stranded. So far the θ defensins have only been found in certain primates. The digestive system of mammals is like a fermentor for 'steady-state' culturing of the natural flora of the respective animals. Thus, the intestinal epithelia can be expected to be centres for antibacterial functions. Groups working with mice and pigs realized this at about the same time. The mouse work was carried out in small-scale and gave first mRNA and genes and after 3 years the first purified intestinal defensin called cryptdin [54].

The pig work produced results the other way round. Viktor Mutt had built a 'factory' for pig intestinal hormones that processed 1 metric tonne per week of frozen small intestine. In Mutt's side fractions we found first cecropin P1 [55], then PR-39 [56] and finally three more peptides derived from known proteins. Cecropin P1 was recently found to be derived from contaminating nematodes [57]. PR-39 belongs to the cathelicidins discussed in the next section. Later pig β-defensin (PBD-1) was isolated from the tongue and the surface of this tissue contains the peptide in antimicrobial concentrations [58]. The work with porcine small intestine also yielded a larger peptide, NK-lysin with 78 residues and three internal S–S bonds. It is the main effector molecule of cytotoxic T and NK cells [59] and the structure can be adopted to kill either bacteria or mammalian cells. When the functions became clear another group isolated the corresponding human molecule, named 'granulysin' [60]. The cytotoxic properties of granulysin may be due to induction of apoptosis [61].

The solution structure of NK-lysin was solved with NMR [62]. It turned out that the hairpin we had imagined had a α-helix structure between each of the disulphide bonds. As NK-lysin was produced only by certain lymphocytes, the barrier function of the intestine is maintained both by different epithelial cells (like the Paneth cells) and by large infiltrations of lymphocytes. A loop segment from the centre of the granulysin was synthesized and showed an activity against mycobacteria, which was encouraging for further work [63].

The genes for human defensins are located on chromosome 8 and the α and β-groups are adjacent and arranged suggesting a duplication. As *in vivo* data appear on the function of different antibacterial peptides, it is becoming evident that defensins and linear peptides work in synergy [64, 65]. The importance of synergism was stressed early on by several investigators (e.g. Elsbach, Lehrer and Zasloff).

Human enteric defensins are well studied. In the intestine the synthesis already starts in the foetus [1] and in adults, induction of HD-5 and HD-6 (which are α-defensins) can be obtained by lipopolysaccharide (LPS) in Paneth cells (review [18]). The processing of HD-5 into two forms (residues 56–94 and 63–94) was recently shown to be carried out by a trypsin originating from the Paneth cells [66].

An elegant study shows that intestinal defensins were found in urine of patients who had their bladder replaced by an artificial one made from their own ileum [67]. Another way to demonstrate the functional capacity of a human gene is to insert it in mice. Such transgenic animals (trangenes) often express several copies of their new gene, but the organ-specific expression is preserved. Mice normally highly sensitive to *Salmonella typhimurium* became resistant when transfected to carry and express human enteric defensin HBD-5 [68].

The human skin is protected by HBD-3, which was first isolated from psoriatic scales and cloned from keratinocytes [69]. The peptide is highly effective against *S. aureus* but also effective against Gram-negative bacteria like *E. coli* and *P. aeuruginosa*. Many defensins are salt-inactivated but HBD-3 is not.

An interesting finding is that isoleucine could induce NF-κB mediated synthesis of β-defensin HBD-2 in a human epithelia cell line [70]. However, care is needed in interpretation of data from cell lines or cultured cells as illustrated by NF-κB regulation of HBD-2 [71].

Cathelicidins and human life

In 1992–93, Zanetti *et al.* discovered a group of cDNA clones which indicated that a number of quite different peptides found in cattle and pigs were attached to a strongly conserved propiece of about 120 residues [72]. As the carrier had been discovered before and given the name cathelin, Zanetti called this family of molecules cathelicidins. There are two recent reviews on cathelicidins [73, 74].

The gene structures for PR-39 from the pig and the human cathelin-LL-37 [75, 76] show that the signal peptide and the entire cathelin part are encoded by the first three exons whilst the processing sequences and the mature effector peptide are encoded by the fourth exon (Fig. 3). In pigs the cathelin-containing genes for protegrins and prophenins are closely linked to the gene for PR-39 [77]. All three polypeptides are mapped to homologous sites on pig chromosome no. 13. The DNA sequences are quite similar including the intron 3, facts that are consistent with an evolution by gene shuffling and horizontal transfer [78].

Several of the cathelicidins are multifunctional and of clear significance for their host. The most remarkable may be PR-39 from pigs. It is a

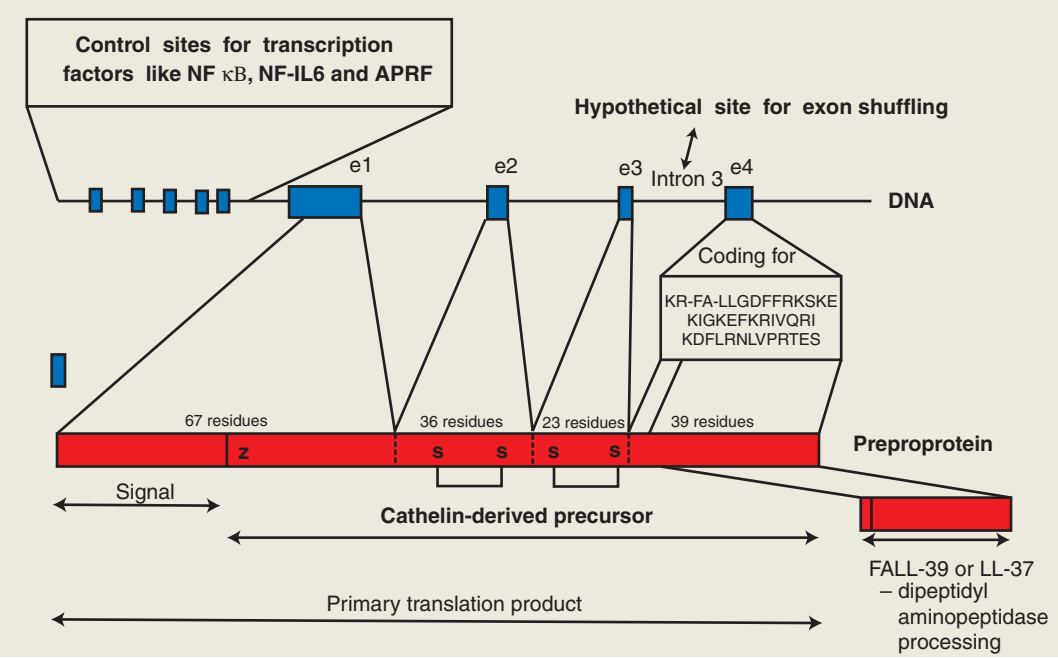

Fig. 3 A cartoon showing the arrangement of the human gene for LL-37 (proFALL-39/hCAP18). The over all structure with three conserved exons (e1–e3) is the same in all cathelicidin genes. The intron between e3 and e4 is rater similar in different animals which facilitates recombination and exon shuffling. The variable part is exon 4, that in pig, cow and sheep can code for different effectors, belonging to the three main groups in Fig. 2. The N-terminal residue in cathelin is pyroglutamate (Z). Thus all precursors contain this N-terminal.

39-residue peptide with 49% proline and 24% arginine and only 11 residues of five other amino acids. Its antibacterial activity showed that it effectively killed bacteria by stopping their DNA and protein synthesis [29]. It was also clear that PR-39 could pass through membranes without any apparent damage. PR-39 has the highest index in Table 2. The molecule can induce the synthesis of syndecans involved in wound healing [79] and interfere with NADPH-dependent redox reactions [80]. The regulation of PR-39 has been carefully worked out [81] and shows that LPS and IL-6 induce mRNA synthesis in isolated granulocytes. PR-39 can also induce angiogenesis [82]. Several companies are now trying to explore PR-39 and its use as a substitute for the heart surgery often needed after an infarct.

The human precursor cathelin-LL-37 (proFALL-39, hCAP-18) was discovered independently by four different groups, but only three reports are published [83–85]. The fourth group was from industry and did not want to publish a structure already registered in GenBank. Our contribution was a cDNA clone that predicted a 30-residue signal, a 100-residue precursor and a C-terminal 39-residue antibacterial peptide [83]. The peptide was synthesized, found potent, and called FALL-39 after the first four N-terminal residues. Chromosome mapping was done in parallel for both PR-39 and LL-37 [75].

The human cathelin precursor of LL-37 has a molecular weight of 16 034 Da, different from 18 kDa implied in the name hCAP-18. The processing of the precursor is not yet finalized. Our first assumption was that the molecule was cleaved after KR, a typical dibasic cleavage site (not yet ruled out). However, removing two N-terminal residues with a dipeptidyl peptidase gave LL-37 [76]. Nevertheless, different views have been expressed favouring granular elastase, protease 3 or bacterial proteases. If the intact precursor is excreted by the granulocytes, enzymes at the site of delivery may control the processing and alternative sites (and enzymes) may offer biological advantages. In addition it is clear that certain bacteria can inactivate LL-37 by proteolytic degradation [86], probably a disease promoting counter defence.

The peptide or the precursor are present in granulocytes, keratinocytes and in certain lymphocytes and monocytes [87] as well as in epithelia such

as the intestine and the skin. In the colon, the expression was linked to cell development [88]. In addition LL-37 and human α-defensins are present both in Vernix Caseosa and amniotic fluid and semen [2, 89]. Table 1 shows that LL-37 has a high index and several investigators have found LL-37 to be multifunctional [90–92] and especially to interact with a receptor [93]. Shigella infections may down-regulate the synthesis of LL-37 [94]. Semen contains both LL-37 and its precursor and the molecule shows affinity for a number of proteins in the semen and may be of importance for human reproduction [89]. An interesting study of the multifunction of LL-37 shows that the peptide in macrophages can reduce levels of tumour necrosis factor (TNF) previous upregulated by LPS [95].

LL-37 is unique to humans; it is the single cathelicidine present in human tissue and so far is found only in rather low concentrations. Thus, investigations of LL-37 have required synthetic peptides and good antibodies for analysis. These tools were used for studies of skin disorders like psoriasis [90] and atopic skin infections caused by *S. aureus* [65]. The clinical importance of LL-37 is clear especially its synergism with the β-defensin HBD-2.

Morbus Kostmann is a severe recessive disorder caused by an unknown mutation in the seventeenth century in northernmost Sweden, and originally defined as an affliction found in descendants of the family described by Kostmann in 1956 [96]. At that time all afflicted children died from bacterial infections during their first year of life (Table 3). Homozygotic parents and siblings remained healthy. Kostmann patients are born with only very few granulocytes and therapy using recombinant G-CSF became possible only after 1990. Thanks to daily injections with this cytokine a few patients with restored granulocyte levels are alive today [97]. However, they are still prone to infections and have to live with antibiotics at hand. In addition, the morphology of granulocytes from treated Kostmann patients are judged as 'activated'. The G-CSF treatment is life saving, but severe oral problems occur as the patients reach adulthood (Fig. 4).

Granulocytes and saliva from four Kostmann patients, one patient with a sporadic neutropenia and one with a Kostmann-like neutropenia were recently investigated [3]. Levels of oxidative burst and of lactoferrin were normal, but Western blot analysis revealed that LL-37 was missing both in granulocytes and saliva of three true Kostmann patients (Fig. 5). One Kostmann patient has been almost cured by a mostly rejected bone-marrow transplantation that restored LL-37 and its precursor. The result correlated with the oral health of the patient. So far this might be the most striking example of the functional need for human antibacterial peptides.

The processing of LL-37 may vary with time and place. However, it should be noted that often there is much more precusor than mature effector peptide (as in Fig. 6). This regulated proteolysis could work like an antibiotic buffer which protects against an overdose and thus has a physiological function. As LL-37 is consumed, the law of mass action predicts that losses are compensated by new hydrolysis of the precursor.

Table 3 Morbus Kostmann: unknown recessive mutation in the seventeenth century

- Syndrome described 1956 by Rolf Kostmann. Family tree
- Diagnosed in infancy: $<0.2 \times 10^6$ neutrophils mL^{-1} blood
- A maturation arrest at the promyelocyte to myelocyte stage
- Lethal – if left untreated (life expectancy <1 year)
- Therapy: antibiotics and rH G-CSF (daily injections)
- Treatment restores neutrophil counts. Patients still suffer from recurrent infections. Severe periodontal disease as adults
- Here we show that Kostmann patients are missing the antibacterial peptide LL-37

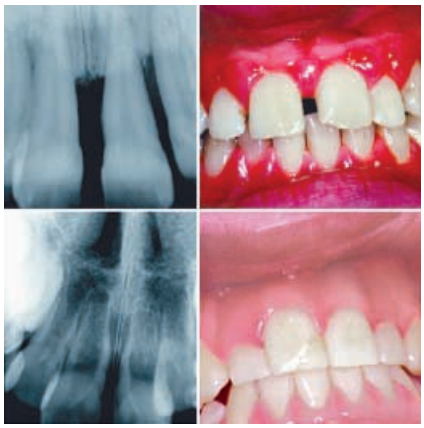

Fig. 4 Upper part: Dental status of Kostmann patient M in the year 2000. X-ray photo (left) and gum status (right) of her front teeth. The pictures were taken at 25 years of age before the patient received supporting bridges. Lower part: The corresponding pictures for her brother.

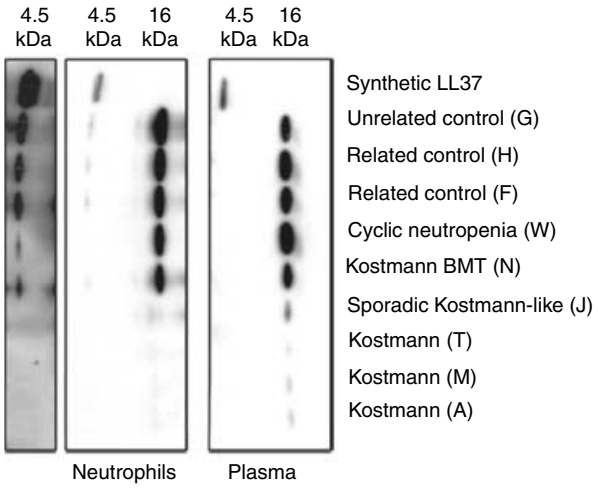

Fig. 5 Western blot analysis of the 4.5 kDa LL-37 in granulocytes a, and the 16 kDa cathelin-LL-37 in granulocytes b, and plasma c, from healthy individuals and Kostmann patients. To estimate the presence of LL-37, the left part of the gel with neutrophil extracts was exposed 100 times longer. Granulocyte extracts (5000 cells per well) were separated on 10–20% SDS-PAGE, blotted onto PVDF membranes, and detected with anti-LL-37 antibody. Plasma samples (2 μL) were analysed similarly. Figures 4 and 5 are taken from reference 3.

In addition, mice have a single cathelicidin with an effector peptide called cathelin-related antimicrobial peptide (CRAMP). The sequences of CRAMP and LL-37 are rather similar (see Fig. 2). Mice with a knockout mutation in the CRAMP gene were found to be abnormally sensitive to Streptococcal A infections [39]. It was also shown that CRAMP is expressed in the salivary gland of mice and that human saliva contains LL-37 and its precursor [98].

Upregulation or storing of pre-made effectors?

Studies of the synthesis of immunoglobulin in human B-cell cultures have revealed a transcription factor (NF-κB) regulating the gene expression of Ig kappa light chain through a κB motif in one of the introns. NF-κB is a heterodimer consisting of the p65 kDa and p50 kDa subunits. The latter is processed from a large precursor of p105, containing ankyrin repeats. The dimer is normally present in the cytoplasm inhibited by IκB, also a molecule with ankyrin repeats. The specificity is derived from

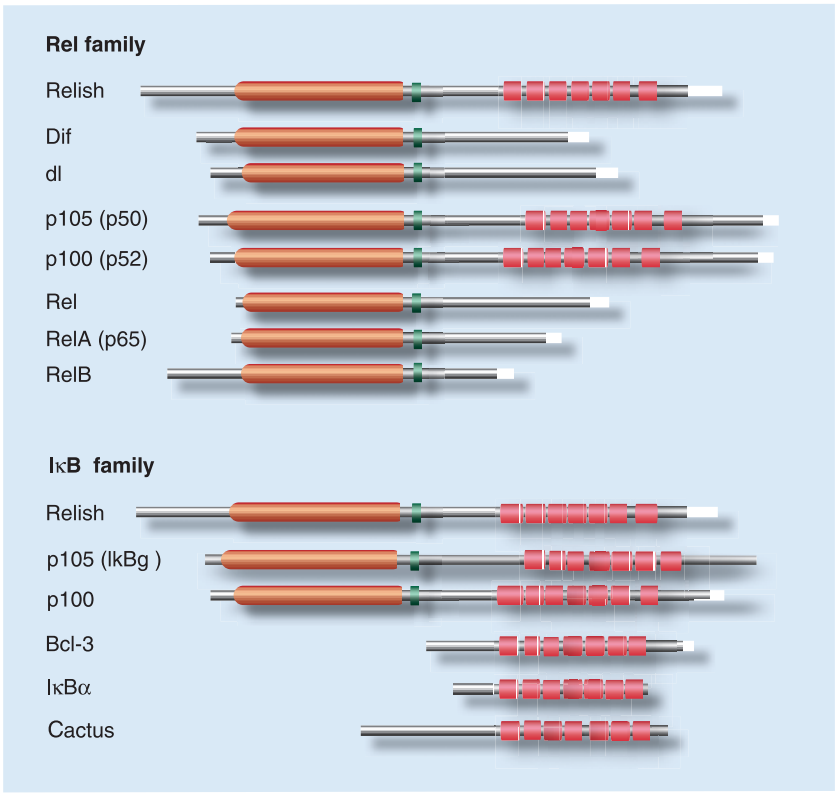

Fig. 6 Cartoon illustrating the similarities between different transcription factors in the NF-κB family and their inhibitors IκB (recoloured and truncated slide of Dan Hultmark). The Rel domain is brownish and followed by greenish domain mediating the nuclear entrance after removing of the inhibitor. In the IκB family the ankyrin repeats are reddish indicating their inhibitory role.

a lock-key mechanism where both the DNA motif and the Rel domains vary. If an inhibitor IκB binds to NF-κB the complex cannot enter the cell nucleus. Activation occurs by phosphorylation of the ankyrin repeats that are then digested by the proteosome. Schematic pictures of Rel molecules and their inhibitors are shown in Fig. 6. At the time of its discovery, the transcription factor may have been regarded as specific. Now NF-κB-like factors are known to be just one part of the 'transcriptosome', a word beginning to be used for the multi-component transcription machinery needed around the RNA polymerase.

Insects have very similar ways of regulating transcription of antibacterial peptides. In Drosophila, at least two signalling pathways are found; these are genetically defined by mutants in genes required, the Toll and Imd genes, respectively [99]. Most investigators still consider Toll and Imd as equally important. However, the transcription factor Relish and the Imd pathway are clearly needed for the expression of all tested antimicrobial peptides [100, 101]. Without a functional Relish gene a fly is killed by as little as a single cell of the otherwise harmless bacterium *Enterobacter cloacae*.

The Relish activation shows clear similarities to the human system. Rel factors, p105 and p100, have compound structures similar to Relish with C-terminal IκB-like domains that are removed by processing. In mammals, the IκB-like halves of the molecules are slowly degraded by the proteosome in a constitutive manner. There may also be links between innate immunity and apoptosis [14].

Vertebrates have closed circulatory systems and a bone marrow for the synthesis of immune cells with their specific effectors. This always involves a transport problem which has to be solved. Granulocytes are loaded with presynthesized peptides, especially α defensins (processed) and LL-37 (as precursor). These cells rapidly move to sites of infection and they have a short half-life. Other blood cells also contain antibacterial peptides, but in lower concentrations [87]. Epithelial cells synthesize their own peptides, LL-37 and the tissue specific β-defensins [102].

What about the receptor concept? The idea is that binding bacterial components to a receptor on a cell surface (cultured cells) should mimic the onset of an infection. The first receptor candidate in flies was Toll [103, 104], previously known from Drosophila development. In vertebrates there are Toll-like receptors (TLR) (TLR-4 needs at least CD-14 as a coreceptor). However, an intriguing recent finding is that in mouse intestinal epithelial cells, TLR-4 recides in the Golgi apparatus rather than on the cell surface [105]. An additional complication is the finding of several peptidoglycan recognition proteins (PGRPs) [106]. The genome of Drosophila encodes 13 PGRPs and one of them, PGRP-LC, mediates signals via the Imd pathway. Both peptidoglycan and LPS induce in this way the synthesis of antibacterial peptides [14]. Four PGRPs are encoded in the human genome, but their role in signalling has not yet been analysed.

Scientists often treat bacterial and viral infections as one and the same phenomenon, which can hardly be biologically correct. Bacteria are recognized and killed by antibacterial peptides without any need for antigen presenting cells. However, there is numerous evidence for links from innate immunity to T cells and to denditric cells (review in Ref. [102]).

It must be stressed that functional peptide concentrations in different mammalian tissues in most cases are too low to be analysed directly, and can only be followed indirectly by PCR or immunological methods. Thus, you cannot know for sure if a given gene is functional, because the level of expression may be below what can be detected. This is relevant to the recent finding of 28 new genes for human β-defensins [107, 108].

Mature granulocytes can be induced to make more of a given antibacterial peptide when stores become depleted [81, 109]. Nevertheless, it appears that the beauty of antibacterial peptides in mammals is their prevention of most bacterial infections, which can occur without any special signalling, as peptides in store are sufficient. Regulation could be built-in to the normal turnover of blood cells and the tissue in question, to homeostasis in general and kept at a steady level sufficient for the control of the normal flora as well as bacterial invaders. This is possible because the size of the normal flora is so much larger than most infections by unwanted bacteria. If newcomers are eliminated within minutes, there is really no need for a specific signalling. Time is saved and the 'defence budget' can be trimmed [110, 111]. But, of course, the signalling has to be there as a safety device. Upregulation may be needed under special normal conditions like conception and delivery or when the load of invading bacteria exceeds the level balanced by normal homeostasis.

Insects, frogs, mice and men

The picture emerging of Drosophila immunity is a system with a surprising rigidity [14]. A very wide range of microorganisms triggers the same major signalling chain, the Relish/Imd-pathway. The discriminatory capacity of the system seems rather limited. The system is also highly conserved. The Toll/Dif-pathway used during differentiation is very similar to the signalling from the human IL-1 and TLR, and there are also parallels between the Imd/Relish-pathways and TNF-α-triggered activation of NF-κB. The PGRPs are important recognition molecules in Drosophila and human homologues have been found that could play a similar role.

Amongst lower vertebrates, different frogs are well investigated [15]. Each species seems to have many (30 or more) antibacterial peptides, especially in the skin and in the intestine. Gene activation is effected by NF-κB-like molecules conserved to the extent that commercial antibodies against human factors could be used. In addition, the inhibitory effects of glucocorticoids on peptide synthesis were first found in frogs [112]. In addition, frogs have rather complete classical immune systems, but there is no clear evidence for its function against bacteria [4].

Interestingly, frogs seems to lack defensins. Of course, negative results are soft facts. However, if correct, during evolution of frogs there must have been a selection against the defensin-type of molecules.

Domesticated animals like cattle, sheep, pigs and goats have been widely used because of easy excess to material. These animals all have multiple cathelicidins (pigs and cattle, 10 each), but it is difficult to investigate their respective functions. However, as the need for *in vivo* experiments grew, the mouse came into focus, although mouse granulocytes turned out to lack α-defensins, which hampers the use of mice as a model for humans. Mice have many enteric defensins (cryptdins) which are well studied [16]. The peptide pattern in the small intestine of germ-free mice is very similar to animals in which the normal flora had been reintroduced [113]. However, similar to humans, mice have only a single cathelicidin and this has been useful for *in vivo* experiments [39].

The conservation of signalling pathways and even recognition molecules like PGRP is understandable: we meet similar bacteria and they all have peptidoglycan cell walls. The system may not have evolved primarily to be a defence against pathogens. Instead it is selected to control the natural flora that otherwise would constitute a threat [11, 111]. In other words it is the peptide antibiotics that make you stay healthy. An ecological balance will always allow a few specialized bacteria (which may have no other niche) to be resistant to the antimicrobial peptides of a host.

The evolution has selected mechanisms of survival for the species, but not for the individual. For some hosts infections may in the end be fatal, but the rate of progression may be slow enough to allow reproduction (such as in TB). Personally, I am not sure whether there is a need for 'self and nonself-recognition' mechanisms involving bacteria. It is possible to avoid self-destruction by selecting effectors for the most universal targets (the bacterial cell wall) that are absent in the host. To accomplish this goal under normal life circumstances we seem to need at least five α-defensins, three β-defensins, LL-37 as well as lysozyme and phospholipase (the latter two perhaps act mostly as cleaning agents). Simplicity is always an evolutionary advantage and a peptide-enzyme based defence is compatible with the concept of a limited defence budget: saving of DNA as well as energy [111].

All human antibacterial peptides are hardly known by now. However, since the human genome is known, it would be possible to design computer searching for new basic peptides with less than 40 residues and potentially forming amphipathic α-helices. However, the search motors available today are not capable of handling short polypeptides.

All animals areas exposed to bacteria are protected not only by endogenous antibacterial peptides, but also by the microbes that already live there, i.e. the normal microflora that is partly self-regulating. The flora has a certain buffer capacity, but if this is exceeded the results may be very serious. *Clostridium difficile* is part of the human intestinal flora, normally only present as a limited number of spores. However, if the flora is damaged by human treatment (like a prolonged antibiotic cure), Clostridia spores mature and the growing bacterium produces a toxin that destroys the mucosal membranes giving severe diarrhoea. So far the best treatment has been to remove what is left of the old flora and replace it by the flora of a healthy nearby donor.

Malaria infections are one of the World's most serious health problems with millions of deaths each

year, mainly amongst children. This was the main reason for sequencing the full genome of the malaria mosquito *Anopheles gambiae* [114]. As the Drosophila genome was available and as innate immunity is better understood in the fly than in any other animal, it was interesting to see how much of the fly data would help in the understanding of the mosquito [115].

The life stages of the malaria parasite have long been known and many attempts have been made to make vaccines that block the liver or the red blood cell forms. However, much fewer details are known about the mosquito parts of the life cycle. In the Anophiles genome there are four defensin and four cecropin genes that are induced by a malaria infection [116]. This finding of cecropin genes is satisfactory from a personal point of view, because one reason to start with Drosophila in 1970 was the long term goal of understanding insect borne diseases. Recently a single-chain immunotoxin with a cecropin-like effector part was shown to kill ookinetes of the malaria parasite *Plasmodium berghei in vitro* [117]. Moreover, mosquitoes with recombinant *E. coli* expressing this immunotoxin in their gut were able to block 95.6% of the malaria transmission in the next blood meal.

When an uninfected mosquito takes a blood meal from a malaria-infected human, some of the parasites enter as gametocytes. In the gut of the mosquito, they begin to mate and form zygotes. The blood meal involves a very complex physiology, needed both for the mating of the parasites and for the egg laying of the host. It also affects the gut bacterial population, which increases and peaks 2 days after a blood meal [118]. A few mosquitoes kill all parasites in a blood meal. They cannot transfer the parasite and are termed 'refractory'. One way to break the infectious cycle would be to alter the blood meal digestion in such a way that it always includes the killing of parasites. When that goal is accomplished it remains to spread the trait in the mosquito population either genetically or by their gut flora or their symbionts.

Antibacterial peptides in medicine – a forecast

It is medically important to understand why people stay healthy. Why does one person fall ill, whilst the one next to him stays well, despite both being exposed to the same microbial environment. When it comes to infections, the classical approach has been to cultivate the suspected pathogen. That approach does not give the correct information: what we need are assays for fast acting immune effectors. Immunological assays with increasing sensitivity and improved accuracy will probably come, but they will only quantify what we already know to exist. Our finding of LL-37 in saliva and its absence in patients with morbus Kostmann [3] indicates that saliva could be a convenient mirror of granulocyte components.

Of the antibacterial peptides already known, will some of them become new drugs? Yes, that seems at present rather likely. For known structures, modifications may be needed in order to obtain adequate patent protection; if not there is hardly any commercial interest. There are several scientists involved in drug development [73, 74, 119] and several peptides are in stage III of clinical trials. In a recent review there is a table listing seven companies involved in the development of antibacterial peptides as drugs [120].

If antibacterial peptides become available on the market – will resistant mutants automatically become a problem? As a forecast, I would say 'probably not' and refer to the following arguments:
1 If bacterial membranes are the targets, experiments carried out indicate that it is quite difficult to isolate mutants with an altered membrane composition. It has been done, but the changes introduced (altered charge) decreased the viability of the mutants to such an extent that they hardly would survive in nature.
2 If the peptide structure (without any modifications) comes from an animal, it does not involve a new selection pressure. This means that the resistance mutations that could occur have already happened. If a natural peptide structure is altered (for patent reasons), argument (1) is still valid, whilst (2) may be weaker.
3 If one is to mimic nature one should always work with a combination of peptides – that is how we stay healthy. A resistant mutant would then have to be altered in such a way that several peptides made from different genes are simultaneously inactivated. This is unlikely ever to happen.

My long-term guess would be that future drugs would be designed to interfere with the synthesis of host peptides in the organ or location desired. Such a

drug may already have been discovered, namely isoleucine. This amino acid can in cell cultures induce the synthesis of HBD-2 [70]. If these results turn out also to be valid for human beings, it would come close to a dream for the patient and the doctor.

Conflict of interest statement

No conflict of interest was declared.

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